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Mary Ann Liebert, Inc.

Optimization of Plasmid Vectors for High-Level Expression in Lung Epithelial Cells

NELSON S. YEW, DONNA M. WYSOKENSKI, KATHRYN X. WANG, ROBIN J. ZIEGLER, JOHN MARSHALL, DAVE McNEILLY, MARIBETH CHERRY, WILLIAM OSBURN, and SENG H. CHENG

ABSTRACT

Nonviral gene therapy approaches use a plasmid vector to express the desired transgene. We have systematically examined several regulatory elements within plasmid vectors that govern gene expression, *e.g.*, the promoter, enhancer, intron, and polyadenylation signal, by constructing a series of plasmids that differed only in the particular sequence element being evaluated. Of the several promoters and polyadenylation signal sequences that were tested, the human cytomegalovirus (CMV) immediate early gene promoter and the addition of polyadenylation signal sequences from the bovine growth hormone (BGH) gene or rabbit β-globin gene produced the highest levels of expression *in vitro*. The inclusion of a hybrid intron 3′ to the promoter further increased expression 1.6-fold. The addition of a region of the CMV enhancer 5′ to several weak promoters increased expression 8- to 67-fold, and co-transfection with a second plasmid encoding a chimeric transcription factor also enhanced expression. On the basis of these results, the CMV promoter, the hybrid intron, and the BGH polyadenylation signal were selected for consistent high level expression *in vitro* and in the mouse lung. However, expression was transient, with greater than 60% loss of activity in the first 7 days. This transient expression was not specific to CMV promoter-containing plasmids, because plasmids containing other heterologous promoters showed a similar profile of transient expression *in vivo*. These comparative analyses begin to provide a basis for the development of optimized expression plasmids for gene therapy of lung diseases.

OVERVIEW SUMMARY

This study reports on our efforts to optimize plasmid vectors for cationic lipid-mediated gene therapy of cystic fibrosis. We have systematically evaluated several regulatory sequence elements within plasmid vectors that affect the level of gene expression *in vitro* and *in vivo*. The cytomegalovirus (CMV) immediate early gene promoter, a chimeric intron, and the bovine growth hormone polyadenylation signal were the sequence elements that resulted in the highest level of expression in cultured cells and in the mouse lung. The incorporation of an enhancer 5′ to several promoters and co-transfection of a second plasmid encoding a transcription factor also increased levels of expression. The transient profile of expression observed in the mouse lung was not specific to plasmids containing the CMV promoter.

INTRODUCTION

CATIONIC LIPIDS HAVE BEEN USED successfully to deliver genes into a variety of different cell types and tissues, and are currently being employed in gene therapy clinical trials (San *et al.*, 1993; Caplen *et al.*, 1995; Sorscher *et al.*, 1994). These lipids are generally comprised of a hydrophobic anchor linked to a positively charged headgroup that binds and condenses the negatively charged DNA (Felgner, *et al.*, 1994; Gao and Huang, 1995). Although the mechanism is not completely understood, the lipid–DNA complex that is formed is able to transfer DNA across the plasma membrane and at least a small fraction is transported into the nucleus. Some of the desirable features of this approach are the minimal immune response to the lipid–DNA complex, the ability to accommodate large transcription units, and the ease of producing large amounts of the components. However, cationic lipid-mediated gene transfer is

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inefficient compared to virus-based vectors. For example, in terms of absolute mass, a hundred- to thousand-fold more DNA is required to achieve a level of expression that is comparable to that of a recombinant adenovirus gene transfer vector (Lee *et al.*, 1996). Because there is a dose-dependent toxicity associated with the use of the lipid–DNA complex, it is advantageous to minimize the amount of complex by optimizing the performance of both the cationic lipid and the plasmid. Recently, several new cationic lipids with increased performance have been synthesized and improved lipid formulations have been developed (Felgner *et al.*, 1994, 1995; Remy *et al.*, 1994; Guy-Caffey *et al.*, 1995; Solodin *et al.*., 1995; Gao and Huang, 1996; Lee *et al.*, 1996; Lewis *et al.*, 1996), but equally important is the optimization of the plasmid vector for high-level expression. A systematic evaluation of the various sequence elements that contribute to high levels of expression in the target cells is the first step in developing an optimal vector.

A major disease target for gene therapy is cystic fibrosis (CF), which is caused by mutations in a single gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan *et al.*, 1989). CFTR transports chloride across the apical membranes of secretory epithelia, and mutations in the protein lead to an electrolyte imbalance. The principal organ affected in CF is the lung, and the defect in CFTR results in a thickened mucus, reduced mucocilliary clearance, and chronic airway infections (Welsh *et al.*, 1995). To develop an optimal vector for expressing CFTR in airway epithelial cells, we evaluated the effects of altering plasmid regulatory elements such as the promoter, enhancer, intron, and polyadenylation signal on expression. The ability of enhancers to increase expression was examined in particular, because these elements can strongly influence transcriptional activity.

In addition to these basic elements, we have also assessed the ability of transactivating factors to increase expression. Regulated inducible expression systems have been described in which a transcriptional activation domain is fused to a regulatory domain such as the tet repressor or progesterone receptor (Gossen and Bujard, 1992; Wang *et al.*, 1994; Shockett *et al.*, 1995; Liang *et al.*, 1996). A small molecule (*e.g.*, tetracycline or a progesterone antagonist) is then used to allow the transcriptional activator to function. Our interest was not in regulation per se, but rather the absolute levels that could be achieved using a transactivator.

A second goal was to increase the duration of transgene expression in the lung. Cationic lipid-mediated gene transfer into the mouse lung only produces short-term expression that rapidly declines within the first 7–10 days (Lee *et al.*, 1996). In an attempt to determine if this was due to the inactivation of the viral CMV promoter used in these studies, other nonviral promoters were tested for their persistence in the mouse lung.

MATERIALS AND METHODS

Construction of plasmids

Most of the plasmids used in this report were derived from pCF1-CAT, whose construction has been previously described (Lee *et al.*, 1996). The promoters were subcloned from plasmids or amplified by the polymerase chain reaction (PCR) from genomic DNA. The human surfactant protein C promoter was a 3.7-kb *Hind* III fragment from hSPC-CFTR (Korfhagen *et al.*, 1990). The human nitric oxide synthase promoter (from −425 to +63 relative to the transcription start site), the human ubiquitin B promoter (from −278 to +74), and the human interleukin-8 promoter (from −335 to +54) were amplified from human genomic DNA (Clontech) by PCR using Pfu Polymerase (New England Biolabs). The human mucin 1 promoter (corresponding to −686 to +31) was a ~730-bp *Eco* RI–*Kpn* I fragment from p-686CAT (Abe and Kufe, 1993). The rat Clara cell CC10 promoter was a 300-bp *Acc* I–*Hind* III fragment from pRtCC10-CAT-2300 (Stripp *et al.*, 1992). The adenovirus E1a promoter was amplified from adenovirus type 2 DNA. For the plasmids containing the bovine growth hormone (BGH) polyadenylation [poly(A)] signal, the promoters were inserted into pCMV(A-N)kan, a derivative of pCMVHI2BGH/kan (Lee *et al.*, 1996) containing a small polylinker. The promoters replaced the CMV promoter and the cDNA for chloramphenicol acetyltransferase (CAT) was inserted into the *Not* I site. For the plasmids containing the SV40 poly(A) signal, the late SV40 poly(A) signal replaced the BGH poly(A) of pCMVHI2BGH/kan and an ampicillin resistance gene replaced the kanamycin resistance gene.

The CMV enhancer placed 5′ to some of the promoters was a 431-bp *Ban* I–*Eco* RI fragment (corresponding to −118 to −522 relative to the transcription start site) from pCMVβ (Clontech). This enhancer was inserted into the *Kas* I site of pCMVHI2BGH/kan. The Rous sarcoma virus (RSV) long terminal repeat (LTR) enhancer (nucleotides 2,365–2,540) was amplified by PCR from pOPI3CAT (Stratagene).

The SV40 19S/16S intron is a 180-bp *Xho* I–*Not* I fragment from pCMVβ. The SV40 19S/16S *Xho* I–*Not* I intron fragment was deleted to form the no-intron vector. The hybrid intron was a 500-bp *Pml* I–*Not* I fragment obtained from pAdβ (Clontech; see also Wong *et al.*, 1985).

The rabbit β-globin polyadenylation signal sequence was synthesized as a 56-bp double-stranded oligonucleotide (Levitt *et al.*, 1989). The sequence was inserted in place of the SV40 poly(A) signal sequence in pCMVHI-CAT. The BGH poly(A) signal sequence was amplified by PCR from pcDNA3/CAT (Invitrogen) and inserted in place of the SV40 poly(A) signal in pCMVHICAT.

The reporter gene encoding the secreted form of human placental alkaline phosphatase (SEAP) was obtained from pBC12/PL/SEAP (Tropix). pCF1-SEAP was constructed by inserting the SEAP cDNA into pCMV(A–N)kan.

The plasmids containing the five GAL4 binding sites were derived from pCF1-SEAP. The five GAL4 binding sites were assembled from four synthetic oligonucleotides. p5GCMV-SEAP was constructed by inserting the five GAL4 binding sites into the *Avr* II site and the SEAP cDNA into the *Not* I site of pCMV(A–N)kan. p5GΔCE-SEAP was constructed by removing a 322-bp *Aat* II fragment (deleting the CMV enhancer from −462 to −141 relative to the transcription start site) from p5GCMV-SEAP and religating. pCAGAL4-VP16 was provided by Dr. Jane Amara (ARIAD Pharmaceuticals). pCMV71, which expresses the cytomegalovirus tegument 71-kD protein from the CMV promoter, was a gift from Dr. Mark Stinski.

Transfection assays

ELM cells, originally thought to be an airway epithelial cell line but later determined to be epithelial-like mouse cells, or CFT1 cells, derived from a CF human tracheal epithelium (Yankaskas *et al.*, 1993), were seeded onto either six-well tissue culture plates at 4×10^5 cells per well or 24-well tissue culture plates at 1×10^5 cells per well. Cells were co-transfected with 1.5 pmol of each CAT construct and 0.6 pmol of pCMVβ as an internal control using the lipid DMRIE:DOPE (Vical, Inc.) at a cationic lipid:DNA ratio of 21 μM:15–30 μM, the molarity of DNA referring to the molarity of nucleotides that comprise the DNA and using an average molecular mass of 330g/mole-nucleotide. Five hours after transfection, the complex was removed from the cells and 1–3 ml of complete CFT1 media (F-12 + 7× medium + 10% FBS) was added to each well. Forty-eight hours after transfection, cells were harvested and CAT protein levels were measured using a CAT ELISA assay (5 Prime-3 Prime). β-Galactosidase (β-Gal) activities were also measured in the same lysates using a chlorophenolred-β-D-galactopyranoside (CPRG) spectrophotometric assay as described previously (Lee *et al.*, 1996). The CAT protein values were divided by the units of β-Gal activity to normalize for potential differences in transfection efficiency between the plasmid DNA preparations of the CAT vectors. An average of five transfections and assays were performed with each plasmid.

Expression from the SEAP gene was quantitated by measuring the enzymatic activity of the secreted SEAP in the cell culture supernatant (Berger *et al.*, 1988). SEAP assays were performed as follows: 0.5–10 μl of cell culture supernatant was sampled 48 hr after transfection and brought up to 100 μl with medium and then transferred to a 96-well tissue culture plate (Costar). The plate was incubated at 65°C for 30 min to inactivate endogenous phosphatase activity, after which 100 μl of 2× SEAP buffer (2 *M* diethanolamine, 1.32 m*M* 4-methylumbelliferylphosphate pH 10) was added and the plate incubated at 37°C for a further 0.5–2 hr. The plate was then read in a fluorimeter with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Serial dilutions of purified human placental alkaline phosphatase (Calbiochem) were used to generate a standard curve.

In vivo *assays*

BALB/c mice were instilled intranasally with 100 μl of lipid GL67:DOPE:DNA (1 m*M*:2 m*M*:4 m*M*) complex as described (Lee *et al.*, 1996). Lungs were harvested 48 hr after instillation and CAT activity in mouse lungs was assayed as described (Lee *et al.*, 1996). A minimum of 4 mice were used for each time point.

RNase protection assay

Cells were transfected with the different CAT plasmids and pCMVβ as an internal control as described above. Total RNA was isolated 48 hr after transfection using RNA STAT-60 (TEL-TEST "B", Inc.) as described by the manufacturer. A total of 8×10^4 cpm of a 241-bp CAT antisense RNA probe and 8×10^4 cpm of a 344-bp β-Gal antisense probe (both probes supplied by the manufacturer, Ambion, Inc.) were mixed with 10 μg of total RNA from each sample in 20 μl of hybridization buffer (Ambion, Inc.). Each sample was denatured at 95°C for 3 min, then incubated at 45°C overnight. The hybridization mixtures were digested with 0.5 units of RNase A and 10 units of RNase T1 at 37°C for 30 min, and the protected double-stranded RNAs were separated on an 8 *M* urea/6% polyacrylamide gel. The protected fragments were quantitated by phosphoimager scanning (Molecular Dynamics).

RESULTS

Effect of different introns on CAT expression

Previous studies in cultured cells and in transgenic mice have shown that the inclusion of heterologous introns in expression plasmids increases expression (Huang and Gorman, 1990; Niwa *et al.*, 1990; Choi *et al.*, 1991). We examined the effect of two introns on expression *in vitro*, one containing the SV40 late viral protein 16S/19S splice donor and acceptor signals and the other a hybrid intron containing a truncated tripartite leader from adenovirus, the splice donor site from the first exon of the tripartite leader, and a splice acceptor site from a mouse immunoglobulin gene (Wong *et al.*, 1985). These introns were inserted into a vector containing the immediate early gene promoter from cytomegalovirus (CMV), the CAT reporter gene, and the SV40 poly(A) signal sequence. Equimolar amounts of each plasmid were transfected into ELM cells, an epithelial-like mouse cell line, and the resultant CAT expression levels compared 48 hr post-transfection. Plasmid vectors containing no intron or containing the SV40 intron gave comparable levels of expression in ELM cells (Fig. 1A). However, the inclusion of the hybrid intron generated 1.6-fold higher levels of CAT expression. The presence or absence of the truncated tripartite leader sequence within the hybrid intron did not have any effect on the observed levels of expression (not shown).

Expression vectors containing different polyadenylation signal sequences

To determine if the origin of the poly(A) signal sequence affected expression, we compared three different poly(A) addition signals, derived from the rabbit β-globin gene, the SV40 late genome, and the BGH gene. The SV40 and BGH poly(A) signals are commonly used in expression vectors, and the β-globin poly(A) signal has been shown to be efficiently recognized in insect and mammalian cells (Levitt *et al.*, 1989; Westwood *et al.*, 1993). The poly(A) signals were inserted in a vector containing the CMV promoter, CAT reporter gene, and the hybrid intron. The vectors containing the BGH (pCMVHIBGH-CAT) or rabbit β-globin poly(A) signal (pCMVHIβglo-CAT) gave the highest level of expression in ELM cells (Fig. 1B), approximately 2.5-fold higher than that observed with the late SV40 poly(A) signal. The relative levels of CAT RNA were also measured directly using a quantitative RNase protection assay. There was a two-fold increase in CAT RNA present in the BGH poly(A) signal vector compared to the SV40 poly(A) signal vector (see Fig. 4B), consistent with the CAT protein results.

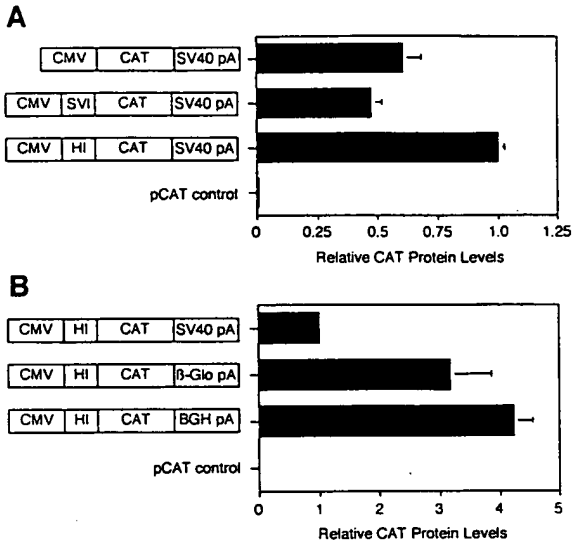

FIG. 1. Effect of different introns (A) and polyadenylation signals (B) on CAT expression. ELM cells were co-transfected with equimolar amounts of each plasmid, and pCMVβ as an internal control using the lipid DMRIE:DOPE and CAT protein levels in cell lysates were assayed 48 hr after transfection. SVI, SV40 19S/16S intron; HI, hybrid intron; SV40 pA, SV40 late polyadenylation signal; BGH, bovine growth hormone polyadenylation signal; β-Glo, rabbit β-globin polyadenylation signal. The data are expressed as mean ± SD (n = 3).

Comparison of vectors containing different promoters

To compare relative promoter strengths, a number of plasmids containing different promoters were constructed and tested *in vitro*. Because the target tissue for CF gene therapy is the lung, a number of promoters from genes known to be expressed in the lung epithelium were analyzed: the surfactant protein C (SPC) promoter (Korfhagen *et al.*, 1990), the human nitric oxide synthase (NOS) promoter (Guo *et al.*, 1995), the human mucin 1 (MUC 1) promoter (Abe and Kufe, 1993), and the rat Clara cell 10-kD protein (CC10) promoter (Stripp *et al.*, 1992). Additional promoters tested included the adenovirus E1a promoter (E1A), used previously in a recombinant adenovirus clinical gene therapy vector (Zabner *et al.*, 1993), the human ubiquitin B promoter (UbB), an abundant protein expressed in all cell types (Baker and Board, 1987), and the human interleukin-8 promoter (IL-8), a cytokine-responsive promoter (Nakamura *et al.*, 1991). The activity of the promoters was compared relative to two CMV promoter vectors, one containing the BGH poly(A) signal (pCF1-CAT) and the other containing the SV40 poly(A) signal (pCMVHICAT). The plasmids were tested in ELM cells and in CFT1 cells, a human airway epithelial cell line derived from a CF patient. Expression from pNOS-CAT and pUbB-CAT were approximately 5% and 20% of pCF1-CAT, respectively, in ELM cells and not above background levels in CFT1 cells. (Fig. 2A). Expression from pCC10-CAT and pE1A-CAT were also low, 2–3% and 5–8% of pCMVHICAT, respectively, in both of the cell lines tested (Fig. 2B). Thus, of the promoters examined, the CMV promoter yielded the highest expression *in vitro*.

Effect of adding a CMV enhancer 5′ to promoters

To determine if adding a segment of the strong CMV enhancer 5′ to a promoter could increase expression, the region of the CMV promoter from −118 to −522 relative to the transcription start site was inserted upstream of the vectors containing the SPC, MUC1, IL-8, and CC10 promoters. In both ELM and CFT1 cells, addition of the enhancer increased expression of the CC10 promoter plasmid by greater than 10-fold (Fig. 2B). The MUC1 and IL-8 promoter plasmids containing the 5′ CMV enhancer also expressed well, approximately 30–36% of pCF1-CAT (Fig. 2A). An exception was the SPC promoter plasmid containing the 5′ CMV enhancer, which expressed at only 3% of the levels of pCF1-CAT (Fig. 2A).

We also tested whether adding the CMV enhancer would affect the responsiveness of the inducible IL-8 promoter to stimulation by cytokines. Inflammatory mediators such as tumor necrosis factor-α (TNF-α) or lipopolysaccharide induce IL-8 expression, and this activation occurs mainly at the level of transcription (Nakamura *et al.*, 1991). The region of the IL-8 pro-

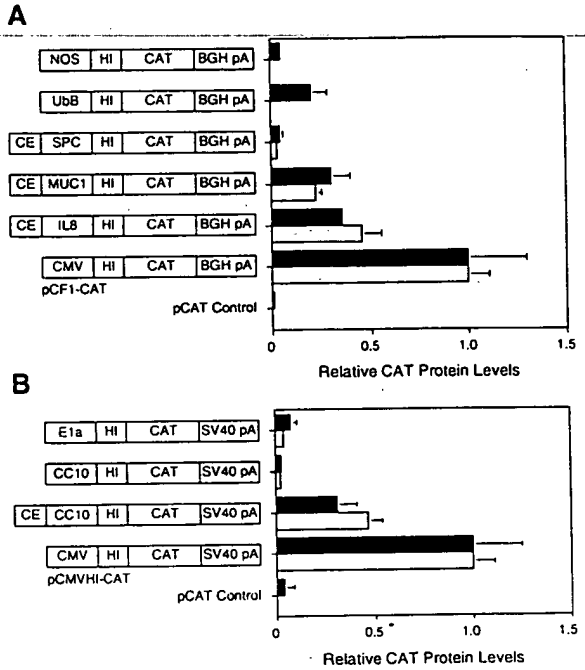

FIG. 2. Comparison of CAT expression from different promoters *in vitro*. ELM cells (solid bars) or CFT1 cells (stippled bars) were transfected as in Fig. 1. CAT ELISA assays were carried out 48 hr after transfection. An average of six assays were performed with each plasmid. CAT protein levels were normalized to pCF1-CAT (in A) or pCMVHICAT (in B). A. Expression from plasmids containing the BGH poly(A) signal. SPC, Surfactant protein C promoter; NOS, nitric oxide synthase promoter; UbB, ubiquitin B promoter; MUC1, mucin 1 promoter; IL8, interleukin-8 promoter; CE, CMV enhancer. pCAT control is a promoterless CAT plasmid. B. Expression from plasmids containing the SV40 poly(A) signal. CC10, Clara cell 10-kD protein promoter; E1a, adenovirus E1a promoter. The data are expressed as mean ± SD (n = 3–12).

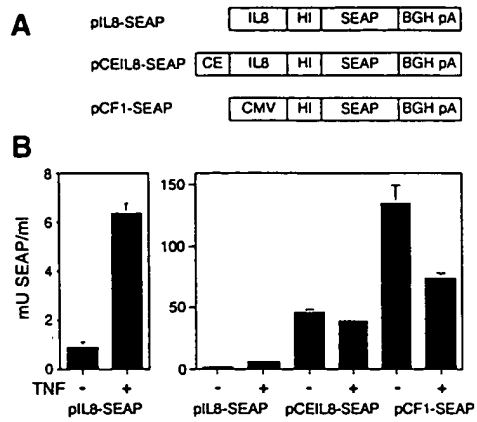

FIG. 3. Effect of an enhancer on the inducibility of IL-8 promoter plasmids. ELM cells were transfected as in Fig. 1. TNF-α (20 ng/ml) was added 24 hr after transfection, and SEAP protein levels in the cell culture media were assayed 24 hr after the addition of TNF-α. A. Diagram of plasmids. IL8, Interleukin-8 promoter; CE, CMV enhancer. B. SEAP activity in the absence or presence of TNF-α. The data are expressed as mean milliunits of SEAP activity per milliliter of cell culture supernatant $\pm$ SD ($n = 3$).

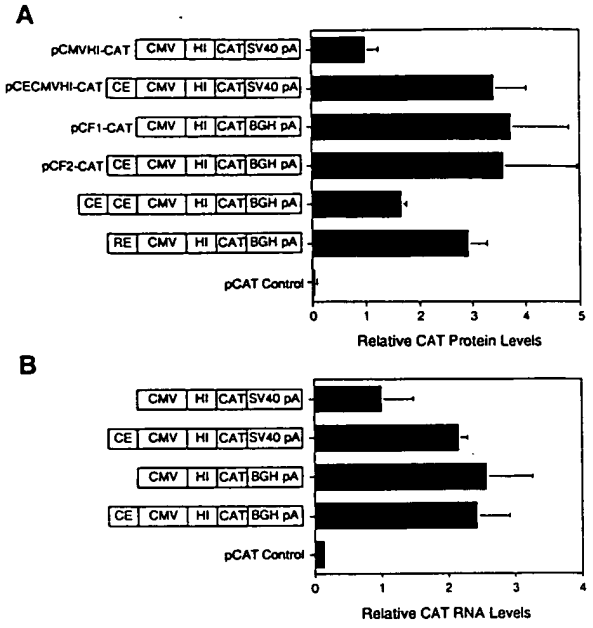

FIG. 4. Effect of a second CMV enhancer region on CAT expression from the CMV promoter. Plasmids were transfected into ELM cells and the cells were harvested 48 hr after transfection. Expression was normalized to pCMVHI-CAT. A. CAT protein levels in cell lysates. CE, CMV enhancer; RE, RSV LTR enhancer. B. Levels of CAT RNA. Total RNA was isolated from the transfected cells and a quantitative RNase protection assay was performed. The data are expressed as mean $\pm$ SD ($n = 3$–9).

moter that has been shown to retain responsiveness to TNF-α (Nakamura *et al.*, 1991) was inserted into the plasmid pIL8-SEAP, which contains the hybrid intron, SEAP reporter gene, and BGH poly(A) signal (Fig. 3A). ELM cells were transfected with pIL8-SEAP and 20 ng/ml of TNF-α was added to the culture media 24 hr post-transfection. SEAP expression was assayed 1 day later. Relative to pCF1-SEAP, expression from pIL8-SEAP was very low (less than 0.5%) but increased approximately 10-fold in response to TNF-α (Fig. 3B). By comparison, expression from pCF1-SEAP decreased nearly two-fold in response to TNF-α. As seen before with the CC10 and E1A vectors, adding the CMV enhancer region 5′ to the IL-8 promoter (to generate pCEIL8-SEAP) increased the unstimulated level of expression, in this case by 67-fold; however, the enhancer also abrogated any additional induction when TFN-α was added (Fig. 3B). Thus, the CMV enhancer appears to supplant the ability of the IL-8 promoter to be induced.

Effect of adding a second CMV enhancer region

To determine if incorporating two CMV enhancers could produce higher levels of expression than one, a second CMV enhancer region (from -118 to -522 relative to the transcription start site) was inserted 186 bp upstream of the CMV promoter and its associated enhancer. The enhancer region was inserted into pCMVHI-CAT (containing the SV40 polyadenylation signal) to form pCECMVHI-CAT, or into pCF1-CAT (containing the BGH polyadenylation signal) to form pCF2-CAT. In the context of the SV40 polyadenylation signal, addition of the second enhancer element increased expression approximately three-fold (Fig. 4A). However, in the context of the BGH polyadenylation signal, adding a second enhancer element did not increase expression (i.e., comparing pCF1-CAT with pCF2-CAT). Eliminating the spacing between the two enhancer regions or reversing the orientation of the second enhancer had no effect (not shown). Adding a heterologous enhancer region, the RSV LTR, to pCF1-CAT also did not increase expression, whereas adding a third CMV enhancer actually decreased expression (Fig. 4A). To determine if the rate-limiting step in expression was at the level of RNA as opposed to translation, a quantitative RNase protection assay was performed. There was a two-fold higher level of CAT mRNA in pCECMVHI-CAT compared to pCMVHICAT, consistent with the expression data (Fig. 4B). There was no difference in the level of CAT mRNA comparing pCF1-CAT to pCF2-CAT, also consistent with the CAT expression results. These data suggest that the rate-limiting step is at the level of mRNA accumulation and not translation.

Increase in expression with chimeric transcription factors

Given the limitations that a strong enhancer can be used to augment expression, we asked if expression could be increased by supplying to the cell a specific transcriptional activator of the promoter. A component of the CMV virion, the 71-kD tegument protein (pp71), has been identified as a potent transactivator of the CMV immediate early genes and was shown to enhance transcription of the CMV promoter by up to 20-fold (Liu and Stinski, 1992). A plasmid expressing the pp71 protein was co-transfected with the CMV promoter-containing pCF1-

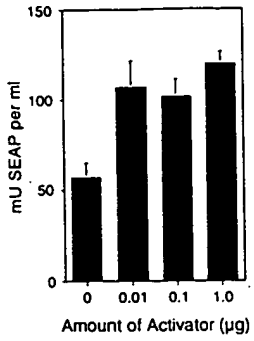

FIG. 5. Effect of the CMV 71-kD tegument protein on SEAP expression from the CMV promoter. ELM cells were co-transfected with 0.5 μg of pCF1-SEAP and 0, 0.01, 0.10, or 1.0 μg of pCMV71. SEAP activity was assayed 48 hr after transfection. The data are expressed as mean ± SD (n = 3).

SEAP. Co-transfection with the pp71-expressing plasmid increased SEAP expression in ELM cells approximately two-fold over that obtained with pCF1-SEAP alone (Fig. 5).

Another transactivator that we examined was the chimeric transcription factor GAL4-VP16. GAL4-VP16 contains the binding domain from the *Saccharomyces cerivisiae* GAL4 gene fused to the activation domain of the herpes simplex virus VP16 protein, a potent activator of viral immediate early gene expression (Sadowski *et al.*, 1988). The plasmid p5GCMV-SEAP was constructed with five GAL4 binding sites inserted directly upstream of the CMV promoter. A second plasmid, pCA-GAL4VP16, contained the GAL4-VP16 activator cDNA under the control of the CMV promoter. Cells were co-transfected with p5GCMV-SEAP and different amounts of the pCA-GAL4VP16. Co-transfection with the GAL4-VP16 activator did not increase expression above that observed in cells transfected with the reporter plasmid alone (Fig. 6A). To determine if the CMV enhancer present in p5GCMV-SEAP was in some way interfering with the GAL4-VP16 activator, a deletion was made in the CMV enhancer between −462 and −141 relative to the transcriptional start site, removing nearly all of the transcription factor binding sites to form p5GΔCE-SEAP. As a result of this deletion, basal expression was low from p5GΔCE-SEAP in the absence of the activator (Fig. 6B). Addition of the GAL4-VP16 activator increased expression by 6- to 40-fold compared with no activator and at the highest amounts of reporter and activator, up to two-fold over the levels attained by pCF1-SEAP.

Comparison of different promoter vectors in vivo

To determine if the activities of the different vectors *in vitro* were reflected *in vivo*, a subset of the plasmids was instilled into the lungs of BALB/c mice. Groups of 4–5 mice were instilled intranasally with 100 μl of cationic lipid/neutral lipid/DNA (1 m*M* GL-67:2 m*M* DOPE:4 m*M* pDNA) complex, and then CAT assays were performed on lungs isolated 48 h after instillation. The expression of different promoter plasmids *in vivo* is shown in Fig. 7A. Expression from pCEMUC1-CAT, which contains the CMV enhancer region 5′ to the Muc 1 promoter (see Fig. 2A) was quite strong, nearly at the level of pCF1-CAT, whereas the activity of pIL8-CAT was very low, consistent with the *in vitro* results. The ubiquitin promoter-containing pUbB-CAT also had extremely low activity, consistent with the results in CFT1 cells.

Plasmids with the BGH poly(A) signal and containing one (pCF1-CAT) or two (pCF2-CAT) CMV enhancers were also tested *in vivo* (Fig. 7B). Approximately equal amounts of CAT (100 ng CAT/100 mg of lung tissue, or approximately 200 ng/lung) were produced from both vectors, consistent with the *in vitro* results.

Persistence of expression in vivo

Previously, we have shown that CAT expression was transient after instillation of GL-67:DOPE:pCF1-CAT into the lungs of BALB/c mice (Lee *et al.*, 1996). Expression declined to approximately 20% of the maximum levels by day 7, although low levels of activity could be detected at day 21. Similar profiles were observed in BALB/c-nu/nu and SCID-treated mice, indicating that the loss of expression was not due to an immune response to the CAT protein (Lee *et al.*, 1996). To de-

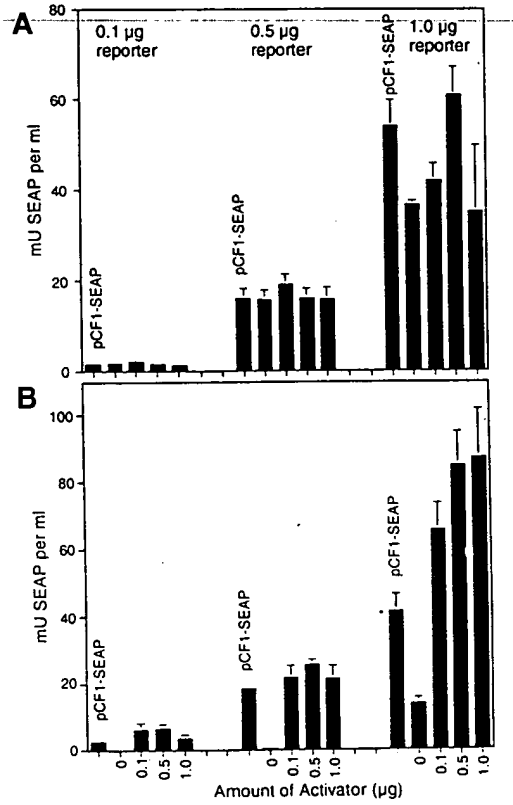

FIG. 6. SEAP expression from CMV promoter vectors in the presence of the chimeric transcriptional activator GAL4-VP16. ELM cells were co-transfected with 0.1, 0.5, or 1.0 μg of p5GCMV-SEAP (A) or p5GΔCE-SEAP (B) and increasing amounts of the GAL4-VP16 activator plasmid. pCF1-SEAP alone (0.1, 0.5, or 1.0 μg) was also transfected into ELM cells for comparison. SEAP activity was measured 48 hr after transfection. The data are expressed as mean ± SD (n = 3).

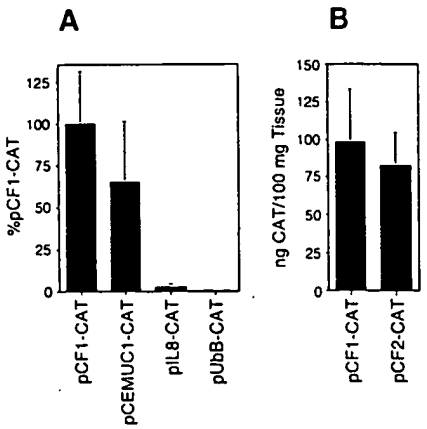

FIG. 7. Comparison of CAT expression from plasmid vectors containing different promoters *in vivo*. BALB/c mice were instilled intranasally with 100 μl of lipid GL-67:DOPE:pDNA complex and CAT assays were performed 48 hr after transfection. A. Comparison of pCF1-CAT, pCEMUC1-CAT, pIL8-CAT, and pUbB-CAT (diagram in Fig. 2A). Data are expressed relative to pCF1-CAT. B. Comparison of pCF1-CAT and pCF2-CAT, expressed as nanograms of CAT per 100 mg of lung tissue. The data are expressed as mean ± SD (n = 4–5).

termine if toxicity associated with the cationic lipid was responsible for the lack of persistence, plasmid DNA alone was instilled into mice. Naked DNA was able to transfect lung cells *in vivo* albeit at a low level. Expression over time, however, was transient and not different from that observed following instillation with lipid–DNA complex (Fig. 8A). This indicated that lipid toxicity was unlikely to be the major cause underlying the transient nature of expression in the lung.

The observed loss of expression may be due to inactivation of the promoter and/or loss of plasmid DNA from the nucleus. To determine if a specific inactivation of the CMV promoter was responsible for the rapid loss of expression, other non-CMV promoters were evaluated *in vivo*. When plotted as the percent of their maximal expression at day 2, the vectors pCF1-CAT, pCEMUC1-CAT, pIL8-CAT, and pUbB-CAT all showed a similar profile of transient expression that declined to 1–16% by day 14 post-instillation (Fig. 8B). The steepest decline in expression occurred within the first 7 days during which the vectors lost 61–92% of their maximal activity, followed by a more gradual decline in expression beyond day 7. Thus, the observed transient expression was not due to inactivation of the CMV promoter specifically.

DISCUSSION

The inefficiency of cationic lipid-mediated gene transfer stems from the number of hurdles that are presented to a plasmid in the path it must take from the outside of the cell into the nucleus. Although a high copy number of plasmids can be transferred into the cell, the majority of the molecules appear to be in large inactive complexes, and a significant rate-limiting step may be entry into the nucleus (Zabner *et al.*, 1995). One solution to this problem is to produce the most efficient expression per plasmid molecule.

The comparison of different introns and polyadenylation signals showed small but significant effects on expression levels. Although reports vary on the effect of introns in plasmid vectors (Huang and Gorman, 1990; Chapman *et al.*, 1991; Liu *et al.*, 1995; Hartikka *et al.*, 1996), we found that in ELM cells the inclusion of the hybrid intron moderately increased expression. We also found that although the SV40 signal sequence is one of the most commonly used polyadenylation signals, it may not be the best choice for optimal expression. The BGH, rabbit β-globin, and late SV40 signal sequences all have been shown to be efficiently recognized (Carswell and Alwine, 1989; Levitt *et al.*, 1989; Goodwin and Rottman, 1992), but there may be cell-specific differences in the rate of cleavage and polyadenylation between the sequences.

The comparison of different promoters showed that the CMV promoter yielded the highest expression in cultured cells and in the mouse lung, consistent with previous studies showing significantly higher activity from viral versus nonviral promot-

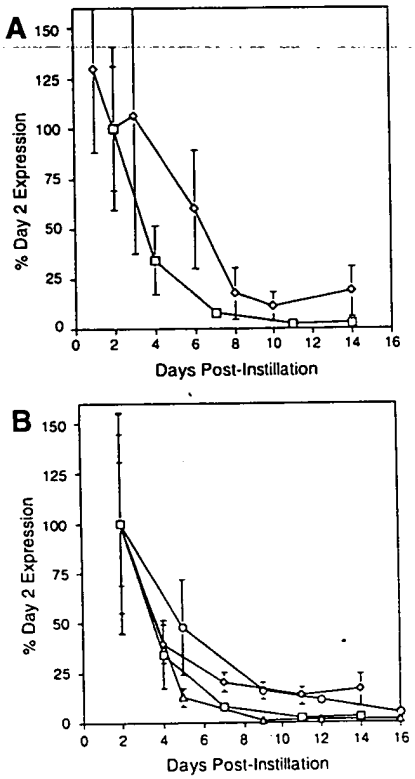

FIG. 8. Lack of persistence of CAT expression *in vivo*. Mice were instilled with 100 μl of either lipid GL-67:DOPE:DNA (0.6 m*M*:1.2 m*M*:3.6 m*M*) complex or plasmid DNA (4 m*M*) alone; then lungs were harvested at different days after instillation. CAT assays were performed after all the lungs had been collected. Data are expressed as percent of day 2 CAT activity levels. A. (◇) pCF1-CAT, (□) lipid GL-67:DOPE:pCF1-CAT. B. Lipid GL-67:DOPE complexed with (△) pCF1-CAT, (◇) pCEMUC1-CAT, (○) pIL8-CAT, (□) pUbB-CAT. The data are expressed as mean ± SD (n = 4–5).

ers, even promoters from genes whose expression is abundant in the particular target tissue (Hartikka *et al.*, 1996). This is not surprising considering the evolution of viruses to maximize production of their own proteins. The other commonly used viral promoters, such as the SV40 promoter and the RSV LTR, have been compared in different cell types (Cheng *et al.*, 1993; Tsan *et al.*, 1995), and although there are exceptions for certain tissues, in most instances the CMV promoter has been found to be the strongest of these viral promoters (Foecking and Hofstetter, 1986). Therefore, the CMV promoter is generally the promoter of choice for high-level expression.

A further increase in expression was achieved by adding a second CMV enhancer region 5′ to the strong CMV promoter, but only in plasmids that contained the SV40 and not the BGH poly(A) signal. Three copies of the CMV enhancer actually decreased expression, a result similar to that observed using multiple copies of the SV40 72-bp repeat enhancer element in a CAT reporter plasmid (Kumar *et al.*, 1986). Inserting four copies versus two copies of the SV40 enhancer element 5′ to CAT increased expression, but inserting greater than four decreased expression, with 10 copies being very inefficient for gene activation (Kumar *et al.*, 1986). Thus, the use of multiple enhancers to increase expression is limited.

Our results suggest that a maximum level of expression can be achieved from a single, nonamplified plasmid. Co-transfection with a second plasmid encoding a transcriptional activator elevated expression above that of a single plasmid as shown using the CMV tegument protein and herpes virus VP16 protein. For a plasmid containing a relatively weak promoter (*e.g.*, the truncated CMV promoter) there was a 6- to 40-fold increase in activity upon co-transfection, with the second plasmid encoding the activator. Endogenous transcription factor binding sites appeared to interfere with this activation, and, in fact, no binding sites upstream of a core promoter were necessary for the VP16 activator to function (not shown). Although the tegument protein or the GAL4-VP16 protein would not be directly applicable to a gene therapeutic because of the predicted immune response to these viral proteins, they demonstrate that moderate gains in expression over even a highly optimized vector can be achieved and it may be possible that co-delivering a cellular transcription factor via an expression plasmid can produce a comparable increase in expression.

These manipulations of regulatory elements do not address the transient nature of expression observed in the mouse lung. The viral CMV promoter was initially thought to be a cause of the problem, because the CMV and SV40 promoters have been shown to be down-regulated by the cytokine interferon-γ, and levels of interferon-γ increase transiently after instillation of the lipid–DNA complex (Harms and Splitter, 1995). The similar transient expression profiles in the mouse lung using plasmids containing the cellular ubiquitin and interleukin promoters indicates that short-term persistence is not unique to the CMV promoter. It is possible that a general repression of promoter activity is occurring that is not specific to CMV.

Loss of plasmid DNA from the nucleus of the transfected cells is another possible cause for the lack of persistence, and we have observed that the levels of plasmid DNA in the instilled mouse lung decline steadily over time (data not shown). Long-term expression from plasmids has been observed in organs such as brain and skeletal muscle (Jiao *et al.*, 1992; Wolff *et al.*, 1992). In the brain, for example, plasmid was found to persist in an episomal form for more than 1 month (Jiao *et al.*, 1992). Short-term expression has been observed in most other organs, highlighting the strong influence the particular target tissue has on the duration of plasmid expression. If loss of plasmid from the nucleus is a significant factor, the incorporation of regulatory elements that increase retention within the nucleus or promote integration into the cell's genome may result in increased persistence.

Although there is a limit to applying results from vectors containing a reporter gene to corresponding vectors containing the transgene of interest, a high level of expression from the optimized pCF1 vector is also observed when CFTR is inserted in place of the reporter gene (data not shown). Further gains may be achieved by maximizing the translation of CFTR through the use of leader sequences and optimal translation initiation sites. It is anticipated that combining an optimized vector with improved lipid formulations and novel strategies to increase persistence will lead to an effective gene therapeutic for cystic fibrosis and other airway diseases.

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Address reprint requests to:
Dr. Nelson S. Yew
Genzyme Corporation
One Mountain Road
Framingham, MA 01701-9322

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